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<p><b>(54) Title: METHOD FOR AMPLIFYING NUCLEIC ACID SEQUENCES</b></p> <p><b>(57) Abstract</b></p> <p>A process for amplifying nucleic acid sequences from a DNA or RNA template which may be purified, or may exist in a mixture of nucleic acids. The resulting nucleic acid sequences may be exact copies of the template, or may be modified. The process has advantages over prior art amplification processes in that it increases the fidelity of copying a specific nucleic acid sequence, and it allows one to more efficiently detect a particular point mutation in a single assay.</p>			
<p>5' —————— 3'   Target DNA</p> <p>5' —————— 3'   1 2   Denature and hybridize to three oligos</p> <p>5' —————— 3'   3  </p> <p>5' —————— 3'   Ligate oligo 1 &amp; 2 and extend oligo 3</p> <p>3' —————— 5'  </p> <p>5' —————— 3'  </p> <p>5' —————— 3'   Repeat Cycle 30 times</p> <p>3' —————— 5'  </p>			

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## METHODS FOR AMPLIFYING NUCLEIC ACID SEQUENCES

### BACKGROUND OF THE INVENTION

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#### 1. Field of the Invention

The present invention relates to a process for amplifying nucleic acid sequences. More specifically, it relates to an improved process for producing nucleic acid 10 sequences from a DNA or RNA template which may be purified, or may exist in a mixture of nucleic acids. The resulting nucleic acid sequences may be exact copies of the template, or may be modified.

15 2. Description of Related Art

In the past, methods have been employed for amplifying nucleic acid sequences wherein both strands of the nucleic acid sequence to be amplified are synthesized by the same method. Such methods are prone to limitations due to the 20 nature of the enzymes utilized in these processes.

In U.S. Patents No. 4,683,195 and 4,683,202, DNA or RNA is amplified by the polymerase chain reaction (PCR). These patents are incorporated herein by reference in their entirety. This method involves the hybridization of an 25 oligonucleotide primer to the 5' end of each complementary strand of the double-stranded target nucleic acid. The primers are extended from the 3' end in a 5' → 3' direction by a DNA polymerase which incorporates free nucleotides into a nucleic acid sequence complementary to 30 each strand of the target nucleic acid. After dissociation of the extension products from the target nucleic acid strands, the extension products become target sequences for the next cycle. In order to obtain satisfactory amounts of the amplified DNA, repeated cycles must be carried out, 35 between which cycles, the complementary DNA strands must be denatured under elevated temperatures.

Traditional polymerases used in this process, such as *E. coli* DNA polymerase I have the limitation of being

inactivated at temperatures necessary for the denaturation of the complementary strands. Thus, between each cycle of synthesis by such polymerases and after the heat denaturation step, a fresh aliquot of enzyme must be added 5 to the reaction mixture so that extension of the primer and synthesis of the complementary strand may occur in the following cycle. This additional step increases the time required for amplification and decreases the ease of amplification which requires multiple steps.

10 In recent years, thermostable DNA polymerases have been discovered and isolated from thermophilic organisms such as *Thermus aquaticus*. Such thermostable polymerases make it possible to add enzyme at the beginning of a series of synthesis and denaturation steps, without the need to add a 15 new aliquot of enzyme after each denaturation step.

20 A potential problem associated with PCR is the hybridization of a primer sequence to regions of the DNA molecule not intended to be amplified. Generally these undesired hybridizations occur because the target sample 25 contains, in addition to the target sequence itself, other sequences with some complementarity to the primer sequences. If the 3' terminal nucleotides of the primer molecule are successfully hybridized to a sequence other than the target sequence, it is possible that primer extension may be successfully initiated by the polymerase 30 enzyme, leading to the generation of an extension product different from the desired target sequence. Under some circumstances, this extension product will undergo exponential amplification, and be erroneously thought to be the desired target sequence.

A method of detecting a specific nucleic acid sequence present in low copy in a mixture of nucleic acids, called ligase chain reaction (LCR), has also been described. European patent application 0 320 308 describes this method 35 and is incorporated herein by reference in its entirety. Target nucleic acid in a sample is annealed to probes

containing contiguous sequences. Upon hybridization, the probes are ligated to form detectable fused probes complementary to the original target nucleic acid. The fused probes are disassociated from the nucleic acid and 5 serve as a template for further hybridizations and fusions of the probes, thus amplifying geometrically the nucleic acid to be detected. The method does not use DNA polymerase.

LCR has disadvantages due to the need for at least four 10 separate oligonucleotide probes for amplification. It also requires that the entire sequence of the target nucleic acid be known. Further, background signal can be caused by target independent ligation of the probes. Since the third probe hybridizes to the first probe and the fourth 15 probe hybridizes to the second probe, the probes, when added in excess, can easily form duplexes among themselves which can be ligated independently of the target nucleic acid.

European Application No. 0 439 182 which is incorporated 20 herein in its entirety by reference discloses a method of improving LCR amplification by providing probes/primers which hybridize to the target nucleic acid wherein one end is modified such that ligation cannot occur until the modified end is corrected. One such modification is the 25 placement of a small gap between the probes preventing ligation of the probes. The gap sequence of the target nucleic acid must be selected such that the DNA sequence is comprised of three or less different nucleotides from the four possible nucleotides. The fourth nucleotide must be 30 the first base complementary to the 5' end of the adjacent probe. The gap is then filled using a DNA polymerase or reverse transcriptase to extend one or more of the probes in a 5' to 3' direction in a target dependent manner to render the probes ligatable. The reaction mixture omits 35 the fourth deoxynucleoside triphosphate complementary to the base at the 5' end of the adjacent probe. Because this

method requires that the gap chosen in the target nucleic acid only contains bases which are complementary to a maximum of three of the deoxynucleoside triphosphates, the method limits the location of the gap on the target nucleic acid and also limits the size of the gap. Further, the method requires four primers. The application also discloses a method of PCR amplification wherein one end of the primer is modified such that the primer is not extendable by a polymerase enzyme. When this modification is removed in a template dependent manner, the primer can be extended. However, this type of PCR requires an additional step of removal of the modification before extension can occur.

In view of the foregoing disadvantages attendant with prior art methods of amplifying nucleic acid sequences, it should be apparent that there exists a need in the art for a method in which the fidelity of amplified sequences can be increased, which allows for the detection of a particular nucleic acid strand, and which allows one to efficiently examine multiple alleles in a single series of amplification steps.

#### SUMMARY AND OBJECTS OF THE INVENTION

The present invention is based on the discovery that certain aspects of LCR and PCR can be used in combination to detect and amplify a target nucleic acid sequence with increased fidelity. Accordingly, in one of its process aspects, the present invention relates to a process for amplifying enzymatically a target nucleic acid sequence contained in a nucleic acid or a mixture of nucleic acids, comprising the steps of:

- a. selecting the target nucleic acid sequence;
- b. providing primers, said primers comprising a first primer which is substantially complementary to a first segment at a first end of the target nucleic acid sequence and a second primer which is substantially

complementary to a second segment at a second end of the target nucleic acid sequence and whose 3' end is adjacent to the 5' end of the first primer and a third primer which is similar to the first end of the target nucleic acid

5 sequence and which is substantially complementary to at least a portion of said first primer;

c. providing at least four different nucleotide bases;

d. hybridizing said first and second primers to

10 the target nucleic acid sequence in a target dependent manner to form a primer-target complex;

e. ligating under conditions such that the adjacent 5' end of the first primer and the 3' end of the second primer will ligate to form a fused amplification

15 product substantially complementary to said target nucleic acid sequence;

f. dissociating said fused amplification product from said target nucleic acid sequence;

g. hybridizing said third primer to said fused

20 amplification product;

h. extending said third primer in the presence of the nucleotide bases under conditions such that an extended amplification product is formed substantially complementary to said fused amplification product;

25 i. dissociating the extended amplification product from the fused amplification product.

In another of its process aspects, the present invention relates to a process for detecting enzymatically a point mutation or allele of a target nucleic acid sequence

30 contained in a nucleic acid or a mixture of nucleic acids using the method disclosed above. One of said primers is comprised of a number of similar oligonucleotide sequences, one of which is exactly complementary to the possible allele or point mutation and each of which oligonucleotides

35 is labeled with a different label. The allele is determined by detecting which labeled oligonucleotide is

contained within the resulting amplification products.

In a third aspect, the present invention relates to a process for amplifying enzymatically a target nucleic acid sequence contained in a nucleic acid or a mixture of 5 nucleic acids comprising the steps of:

- a. selecting the target nucleic acid sequence;
- b. providing primers, said primers comprising a first primer which is substantially complementary to a first segment at a first end of the target nucleic acid 10 sequence and a second primer which is substantially complementary to a second segment at a second end of the target nucleic acid sequence said second segment being spaced from said first segment and a third primer which is similar to the first end of the target nucleic acid 15 sequence and which is substantially complementary to a portion of said first primer;
- c. providing at least four different nucleotide bases;
- d. hybridizing said first and second primers to 20 the target nucleic acid sequence in a target dependent manner to form a primer-target complex;
- e. extending a 3' end of the second primer in the presence of the nucleotide bases under conditions such that an extended second primer is formed wherein the 3' end of 25 the extended second primer terminates at a base adjacent to a 5' end of the first primer;
- f. ligating the ends of the first and second extended primers under conditions such that said first and said extended second primers will form a fused 30 amplification product substantially complementary to said target nucleic acid sequence;
- g. dissociating said fused amplification product from said target nucleic acid sequence;
- h. hybridizing said third primer to said fused 35 amplification product;
- i. extending said third primer in the presence of

the nucleotide bases under conditions such that an extended amplification product is formed substantially complementary to said fused amplification product; and

5       j.     dissociating the extended amplification product  
from the fused amplification product.

In one of its product aspects, the present invention relates to a kit for amplifying a target nucleic acid sequence contained in a nucleic acid or a mixture of nucleic acids comprising: first, second and third primers; 10 a ligating enzyme; a polymerizing enzyme; and at least four nucleotides.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 depicts one embodiment of the method of DNA 15 amplification/detection as set forth herein.

Figure 2 is a printout from a Phosphor Imager of a scanned acrylamide gel. The arrow indicates the resulting higher molecular weight amplification products.

Figure 3 depicts another embodiment of the method of DNA 20 amplification/detection as set forth herein.

Figure 4 shows the sequence of the multidrug resistance gene (MDR-1) (SEQ ID NO:1).

DESCRIPTION OF THE PREFERRED EMBODIMENTS

25       Prior to discussing this invention in detail, the following terms will first be defined:

The "target nucleic acid" or "target nucleic acid sequence" suitable for use in the present invention may be taken from DNA or RNA, and it may be isolated or present in 30 samples which contain nucleic acid sequences in addition to the target nucleic acid sequence to be amplified. The target nucleic acid sequence may be located on a nucleic acid strand which is longer than the target nucleic acid sequence. In this case, the ends of the target nucleic acid 35 sequence are the boundaries with the unselected nucleic acid sequence and the target nucleic acid sequence.

The target nucleic acid sample may be obtained synthetically, or can be isolated from any organism by methods well known in the art. Particularly useful sources of nucleic acid are derived from tissues or blood samples 5 of an organism, nucleic acids which are present in self-replicating vectors, and nucleic acids derived from viruses and pathogenic organisms such as bacteria and fungi. Also particularly useful for the present invention are target nucleic acid sequences which are related to 10 disease states, such as those caused by chromosomal rearrangement, insertions, deletions, translocations and other mutations, those caused by oncogenes, and those associated with cancer.

The term "selected" means that a target nucleic acid 15 sequence having the desired characteristics is located and probes are constructed around appropriate segments of the target sequence.

The term "probe" or "primer" has the same meaning herein, namely, an oligonucleotide fragment which is single 20 stranded. The term "oligonucleotide" means DNA or RNA.

A probe or primer is "substantially complementary" to the target nucleic acid sequence if it hybridizes to the sequence under renaturation conditions so as to allow target dependent ligation or extension. Renaturation 25 depends on specific base pairing between A-X (where X is T or U) and G-C bases to form a double stranded duplex structure. Therefore, the primer sequence need not reflect the exact sequence of the target nucleic acid sequence. However, if an exact copy of the target nucleic acid is 30 desired, the primer should reflect the exact sequence. Typically, a "substantially complementary" primer will contain at least 70% or more bases which are complementary to the target nucleic segment. More preferably 80% of the bases are complementary and most preferably 90% of the 35 bases are complementary. Generally, the primer must hybridize to the target nucleic acid sequence at the end to

be ligated or extended to allow target dependent ligation or extension.

The primers may be RNA or DNA, and may contain modified nitrogenous bases which are analogs of the normally incorporated bases, or which have been modified by attaching labels or linker arms suitable for attaching labels. Inosine may be used at positions where the target sequence is not known, or where it may be degenerate. The oligonucleotides must be sufficiently long to allow hybridization of the primer to the target nucleic acid and to allow amplification to proceed. They are preferably 15 to 50 nucleotides long, more preferably 20 to 40 nucleotides long, and most preferably 25 to 35 nucleotides long. The nucleotide sequence, content and length will vary depending on the sequence to be amplified.

It is contemplated that a primer may comprise one or more oligonucleotides which comprise substantially complementary sequences to the target nucleic acid sequence. Thus, under less stringent conditions, each of the oligonucleotides would hybridize to the same segment of the target nucleic acid. However, under increasingly stringent hybridization conditions, only that oligonucleotide sequence which is most complementary to the target nucleic acid sequence will hybridize. The stringency of conditions is generally known to those in the art to be dependant on temperature, solvent and other parameters. Perhaps the most easily controlled of these parameters is temperature and since the conditions here are similar to those of PCR, one skilled in the art could determine the appropriate conditions required to achieve the level of stringency desired.

Oligonucleotide primers or oligonucleotide probes suitable for use in the present invention may be derived by any method known in the art, including chemical synthesis, or by cleavage of a larger nucleic acid using non-specific nucleic acid-cleaving chemicals or enzymes, or by using

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site-specific restriction endonucleases.

The primers may be prepared using the  $\beta$ -cyanoethyl-phosphoramidite method or other methods known in the art. A preferable method for synthesizing oligonucleotide primers 5 is conducted using an automated DNA synthesizer by methods known in the art. Once the desired oligonucleotide primer is synthesized, it is cleaved from the solid support on which it was synthesized, and treated, by methods known in the art, to remove any protecting groups present. The 10 oligonucleotide primer may then be purified by any method known in the art, including extraction and gel purification. The concentration and purity of the oligonucleotide primer may be examined on an acrylamide gel, or by measuring the optical densities at 260 and 280 15 nm in a spectrophotometer.

In order for the ligase to ligate the oligonucleotide primers, the primers used in the present invention are preferably phosphorylated at their 5' ends. This may be achieved by any method known in the art, but is preferably 20 conducted with the enzyme T4 polynucleotide kinase. The oligonucleotides can be phosphorylated in the presence of unlabeled or labeled ATP. In order to monitor the amplification process, labeled ATP may be used to phosphorylate the primers. Particularly preferable is 25  $[\gamma-^{32}\text{P}]$  ATP.

The oligonucleotide primers may alternatively be labeled with any detectable marker known in the art, including other radioactive nuclides such as  $^{35}\text{S}$  or  $^{3}\text{H}$  and the like, 30 fluorescent markers such as fluorescein, rhodamine, Texas red, Lucifer yellow, AMCA blue and the like, or with enzymatic markers which may produce detectable signals when a particular chemical reaction is conducted, such as alkaline phosphatase or horseradish peroxidase. Such enzymatic markers are preferably heat stable, so as to 35 survive the denaturation steps of the amplification

process. Primers may be indirectly labeled by incorporating a nucleotide covalently linked to a hapten or other molecule such as biotin to which a labeled avidin molecule may be bound, or digoxigenin, to which a labeled 5 anti-digoxigenin antibody may be bound.

Primers may be labeled during chemical synthesis or the label may be attached after synthesis by methods known in the art. The method of labeling and the type of label is not critical to this invention.

10 It is contemplated that the probes or primers may be modified. For example the hydrolysis of a primer by 5' to 3' exonuclease associated with polymerase may be prevented by placing a phosphorothioate group between the last nucleotides of the 5' end of the primer. The extension of 15 a primer by polymerase can be blocked by placing a dideoxynucleotide or a phosphate group at the 3' end. Alternatively, the extension of a primer may be blocked by placing an arabinosyl nucleotide at the 3' end of the primer which blocks extension by polymerase but allows 20 ligation of the primer to another primer.

The term "the four different nucleotide bases" shall refer to deoxythymidine triphosphate (dTTP); deoxyadenosine triphosphate (dATP); deoxyguanosine triphosphate (dGTP); and deoxycytidine triphosphate (dCTP), when the context is 25 DNA, but shall refer to uridine triphosphate (UTP); adenosine triphosphate (ATP); guanosine triphosphate (GTP); and cytidine triphosphate (CTP) when the context is RNA. Alternatively, dUTP, dITP, rITP or any other modified base may replace one of the four nucleotide bases or may be 30 included along with the four nucleotide bases in the reaction mixture so as to be incorporated into the amplified strand. The amplification steps are conducted in the presence of at least the four deoxynucleoside triphosphates (dATP, dCTP, dGTP and dTTP) or a modified 35 nucleoside triphosphate to produce a DNA strand, or in the presence of the four ribonucleoside triphosphates (ATP, ,

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CTP, GTP and UTP) or a modified nucleoside triphosphate to produce an RNA strand from extension of the oligonucleotide which acts as a primer.

Where the presence of a particular mutation or allele is 5 to be detected by the methods of this invention, one of the oligonucleotide primers may comprise a set of oligonucleotide fragments, each differing in sequence and each labeled by a different method. That oligonucleotide fragment which is exactly complementary to the target DNA 10 sequence will be detected by the presence of that label in the amplification products. In this case, each oligonucleotide fragment may be labeled as described above.

#### Utility

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##### First Embodiment

In a first embodiment, the target nucleic acid is described as single stranded. However, this should be understood to include the case where the target is actually 20 double-stranded but is simply separated from its complementary strand prior to hybridization with probes/primers. Primers one and two, together, are substantially complementary to the target nucleic acid sequence and hybridize to adjacent regions of the target 25 nucleic acid strand such that upon hybridization of the two primers to the target nucleic acid strand the 5' end of the first primer is adjacent to the 3' end of the second primer. The 3' end of the first primer is substantially complementary to the 5' end of the target nucleic acid 30 sequence and the 5' end of the second primer is substantially complementary to the 3' end of the target nucleic acid sequence. The 5' end of the first primer is ligated to the 3' end of the second primer using ligase to 35 create a fused amplification product in a double stranded complex. The fused primer is dissociated from the target nucleic acid.

The third primer is substantially complementary to all or at least a portion of the first primer and is similar to the 5' end of the target nucleic acid. The third primer may be smaller than the first primer or it may be larger

5 than the first primer and also be substantially complementary to a portion of the second primer. The third primer is hybridized to the fused amplification product and extended by polymerase in the presence of at least four different nucleotide bases to form an extended

10 amplification product which is substantially complementary to the fused amplification product. This comprises the first cycle.

Subsequently the double stranded complexes are dissociated. The oligonucleotide primers (1 and 2) are

15 hybridized to the target nucleic acid sequence and the extended amplification product from the first cycle. Primer 3 is hybridized to the fused amplification product. Extension and ligation occur as before and the process can be repeated.

20 It is contemplated that the 3' end of the second primer may be modified to block the extension of the second primer by polymerase while still allowing ligation of the 3' end of the second primer to the 5' end of the first primer. Such modification may be, for example, the placement of an

25 arabinosyl nucleotide at the 3' end of the second primer. Methods for the chemical synthesis of DNA oligomers containing cytosine arabinoside are known in the art (Beardsley, Nucl. Acid. Res. (1988) 16:9165-9176). Such a modification does not need to be removed prior to the

30 ligation of the first and second primers.

Alternatively, it is also contemplated that the 5' end of the first primer can be modified to prevent the hydrolysis of the primer by a 5' to 3' exonuclease associated with a polymerase. Such a modification may be,

35 for example, the placement of a phosphorothioate group between the last nucleotides of the 5' end of the first

primer. Methods for the chemical synthesis of phosphorothioate containing primers is known in the art (Ott and Eckstein, Biochemistry, (1987) 26:8237-8241). Such a modification does not need to be removed prior to 5 ligation of the first and second primers.

It is further contemplated that extension of the first primer can be prevented without affecting the ligation of this primer by modifying the 3' end of the primer with a dideoxynucleotide or a phosphate group. The method for 10 producing this modification is known in the art (Markiewicz and Wyrzykiewicz Nucl. Acid. Res. (1989) 17:7149-7158).

It has been found that the process can be conducted sequentially without isolation or purification of the products or removal of the excess reagents. Accordingly, 15 this will allow the entire process to be conducted in a single reaction medium (e.g. a test tube).

It is understood that the single strand variation is a more specialized version of the double strand variation. If the target nucleic acid is double stranded some of the 20 third primers will hybridize to the second complementary strand and the first and second primers will hybridize to the first strand. The extension and ligation from the first strand will proceed as described above. Some of the third primers will also be extended in a target specific 25 manner complementary to the second strand. After dissociation of the extended third primer and the second strand, at least some of the first and second primers will hybridize to the extended third primer and at least some of the third primer will again hybridize to the second strand.

30 Where the target nucleic acid amplified by ligation of the first and second primers and extension of the third primer is to be detected, one or all of these primers may be labeled using a marker as described above to render the amplified target nucleic acid detectable or by conducting 35 the extension of the third primer in the presence of a labeled base, or a base which is activated for labeling.

Alternatively, where the target nucleic acid is double stranded, both amplified strands may be labeled with different detectable markers: the first strand may be labeled by labeling the third primer with a particular 5 marker; and the second strand may be labeled by labeling the first and/or second primer.

In the case where the presence of a particular point mutation or allele is to be detected, one primer comprising a mixture of oligonucleotides is added to the nucleic acid 10 sample. Each oligonucleotide may be labeled with different, separately detectable markers, so that information regarding the presence of a particular mutation or allele may be obtained in a single step. The oligonucleotide which is exactly complementary to the 15 target sequence will be included in the amplification product whereas the other oligonucleotides will not and its presence detected by determining which label is included in the product.

The amplification reaction is optimally conducted with 20 an excess of primers at a ratio of oligonucleotide primers:target of approximately  $10^7$  to  $10^3$ :1, more preferably approximately  $10^4$ :1. It is contemplated that adjusting the molarity of the primers will maximize the efficiency of the process.

25 The buffer used for amplification is preferably in a pH range of about 7.5-8.5, more preferably about 8-8.5, and most preferably about 8.0.

#### Second Embodiment

30 In a second embodiment, the target nucleic acid is described to be single stranded. However, this should be understood to include the case where the target is actually double stranded, but is simply separated from its complementary strand prior to hybridization with the 35 probes/primers.

The target nucleic acid is hybridized to two primers.

The first primer is substantially complementary to the 5' end of the target nucleic acid sequence and the second primer is substantially complementary to the 3' end of the target nucleic acid sequence. The primers (primers one and 5 two) hybridize to regions of the target nucleic acid strand such that upon hybridization of the two primers to the target nucleic acid strand the 5' end of the first primer is spaced from the 3' end of the second primer. The size 10 of the space or gap between the primers is determined by the ability of a polymerase or transcriptase to extend the second primer such that the newly added 3' end of the second primer is directly adjacent to the 5' end of the first primer. Preferably, but not necessarily, the size of 15 the gap or space is sufficiently long such that at least four different nucleotides would be required by the polymerase or transcriptase in order to extend the second primer to "fill in" the gap.

The 3' end of the second primer is extended by 20 polymerase or transcriptase in the presence of the four nucleotide bases. The 5' end of the first primer is then ligated to the new 3' end of the second extended primer to form a double-stranded complex comprising the target nucleic acid and an extended fused primer.

The double stranded complex is dissociated and a third 25 primer is hybridized to the extended fused primer. The third primer is substantially complementary to all or a portion of the first primer and is similar to the 5' end of the target nucleic acid sequence. The 3' end of the third primer is extended by polymerase or transcriptase to form a 30 double-stranded complex. The double-stranded complex is dissociated and the cycle repeated until the target nucleic acid is amplified.

It is contemplated that the process can be conducted 35 sequentially without isolation or purification of the products or removal of the excess reagents. Accordingly, this will allow the entire process to be conducted in a

single reaction medium (e.g. a test tube). Further, because the gap between the primers can be any size, the method is not limited to a particular DNA sequence and extension of the third primer can proceed in the presence 5 of four nucleotides.

It is understood that the single strand variation is a more specialized case of the double strand variation wherein there are four primers and the first and second primers are substantially complementary to the first strand 10 of the target nucleic acid and the third and fourth primers are substantially complementary to the second strand of the target nucleic acid. The third primer being substantially complementary to at least a portion of the first primer and the fourth primer being substantially complementary to at 15 least a portion of the second primer. The extension and ligation of the third and fourth primers occurs as described above for the first and second primers.

It is contemplated that the 5' end of the first primer (and the 5' end of the fourth primer, where the nucleic 20 acid is double stranded) can be modified to prevent the hydrolysis of the primer by a 5' to 3' exonuclease associated with the polymerase. Such a modification may be, for example, the placement of a phosphorothioate group between the last nucleotides of the 5' end of the first or 25 fourth primers. Methods for the chemical synthesis of phosphorothioate containing primers is known in the art (Ott and Eckstein, Biochemistry, (1987) 26:8237-8241). Such a modification does not need to be removed prior to ligation of the first and second primers.

30 It is further contemplated that extension of the first and fourth primers can be prevented without affecting the ligation of these primers by modifying the 3' end of the primers with a dideoxynucleotide or a phosphate group. This method of producing this modification is known in the 35 art (Markiewicz and Wyrzykiewicz Nucl. Acid. Res. (1989) 17:7149-7158).

Where the target nucleic acid amplified is to be detected, one or all of these primers may be labeled as described above to render the amplified strand detectable. Alternatively the strand may be labeled by conducting the 5 extension of the second or third primer in the presence of a labeled base, or a base which is activated for labeling.

In the case where one primer comprises a mixture of oligonucleotides to detect the presence of a particular sequence, each of the oligonucleotides may be labeled with 10 different, separately detectable markers, so that information regarding each mutation may be obtained in a single step.

The amplification reaction is optimally conducted with an excess of primers at a ratio of oligonucleotide 15 primers:target of approximately  $10^7$  to  $10^3$ :1, more preferably approximately  $10^4$ :1. It is contemplated that adjustment of the molarity of the primers will maximize the efficiency of the process.

The buffer used for amplification is preferably in a pH 20 range of about 7.5-8.5, more preferably about 8-8.5, and most preferably about 8.0.

If the target nucleic acid is double stranded, the strands should be separated so that they can be used individually. This separation can be accomplished by any 25 suitable denaturation method including physical, chemical or enzymatic means, each of which are well known in the art.

In either of the above embodiments, the amplification reaction will involve a series of steps. The reaction may 30 be either a two step process [i.e. 1) hybridization/extension/ligation followed by 2) denaturation] or a three step process [1) hybridization; 2) extension/ligation and 3) denaturation]. These steps may be carried out manually, but they are preferably conducted 35 in an automated thermal cycler.

Hybridization is generally conducted at a temperature of

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approximately 50-75°C for a period of 0.5-2 minutes, more preferably at 60-70°C for a period of 1-1.5 minutes, and most preferably at about 63-68°C for about 1 minute. The extension/ligation or the hybridization/extension/ligation 5 steps are generally conducted at a temperature of approximately 60-80°C for a period of 0.5-5 minutes, more preferably at 68-78°C for a period of 2-4 minutes.

The conditions and reagents which make possible the preferred enzymatic ligation step are generally known to 10 those of ordinary skill in the art and depend directly on the type of ligase used. The "ligating enzyme" may be any enzyme known in the art to ligate nucleic acid sequences, including T4 ligase, but it is preferably a ligase stable at temperatures of approximately 0-95°C, such as AMPLIGASE 15 (Epicentre Technologies, Madison Wisconsin) and Taq ligase (New England Biolabs, Beverly, Massachusetts). Absent a thermally stable ligase, the ligase must be added again each time the cycle is repeated. Approximately at least 1 units of ligating enzyme/picomole of oligonucleotide is 20 used. One unit is defined as the amount required to catalyze the ligation of 50% of the cos sites in one microgram of BstE II digested bacteriophage λ DNA in fifteen minutes at 45°C.

The "polymerase" may be any enzyme capable of 25 polymerizing an RNA or DNA strand, including E. coli DNA polymerase I, the Klenow fragment of E. coli DNA polymerase I, T4 DNA polymerase, RNA polymerase or reverse transcriptase. In general, the primer is extended by the polymerase in a target dependent manner, for example, under 30 conditions such that a nucleic acid strand is formed complementary to the nucleic acid sequence to which the primer is hybridized. Preferably, the polymerizing enzyme is stable at temperatures of approximately 0-95°C, such as Taq DNA polymerase (Perkin-Elmer Corporation, Norwalk, 35 Connecticut). Absent a thermally stable polymerase, the

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polymerase must be added again each time the cycle is repeated. At least approximately 0.05 units of polymerizing enzyme (as defined by the manufacturer)/picomole of oligonucleotide is used.

5 Extension of a primer by polymerase or transcriptase proceeds in a 5' to 3' direction and requires the addition in adequate amounts of at least the four nucleotide bases in the reaction mixture.

10 After extension of the primers, it is necessary to separate the nucleic acid strands. The strand separation can be accomplished by any suitable denaturing method including well-known physical, chemical or enzymatic means. For example, one physical method of separating the strands of the nucleic acid involves heating the nucleic acid until 15 it is completely denatured. Typical heat denaturation is generally conducted at a temperature of approximately 85-110°C, more preferably at 90-100°C, and most preferably at about 92-96°C for a period of at least about 0.5 minutes. One skilled in the art would understand how to 20 modify the temperatures and times so as to optimize the results obtained with different oligonucleotide primers. Alternatively, denaturation can be achieved by other methods known in the art. One such method is by the introduction of a nucleic acid-unwinding enzyme such as 25 helicase.

The reaction is stopped by any method known in the art, such as with a buffer containing a high percentage of denaturant such as formamide, EDTA or by freezing. The products can then be analyzed by any method, but 30 electrophoresis on a polyacrylamide gel is preferable. Preferably, the samples are boiled before loading on the gel to eliminate any secondary structures. The gel may then be dried and placed against autoradiographic film or phosphor screen when the oligonucleotides or amplified 35 strands contain radioactive nuclides. The gel may also be blotted and probed with a probe specific to the region

amplified.

The primer may be labeled with a detectable marker by any method known in the art. A preferred method for labeling primers is by end labeling. Primers may be 5 labeled during chemical synthesis by substitution of the  $^{31}\text{P}$  atoms in the phosphate groups with  $^{32}\text{P}$ . The substituted nucleotide may be directly labeled or contain a linker arm for attaching a label, or may be attached to a hapten or other molecule to which a labeled binding molecule may bind 10 (Boehringer Mannheim, Indianapolis, Indiana). Suitable direct labels include radioactive labels such as  $^{32}\text{P}$ ,  $^{3}\text{H}$ , and  $^{35}\text{S}$  and non-radioactive labels such as fluorescent markers, such as fluorescein, Texas Red, AMCA blue, lucifer yellow, rhodamine, and the like; cyanin dyes which are 15 detectable with visible light; enzymes and the like.

Fluorescent markers may alternatively be attached to nucleotides with activated linker arms. Primers may be indirectly labeled by the methods disclosed above, by incorporating a nucleotide covalently linked to a hapten or 20 other molecule such as biotin or digoxigenin, and performing a sandwich hybridization with a labeled antibody directed to that hapten or other molecule, or in the case of biotin, with avidin conjugated to a detectable label. Antibodies and avidin may be conjugated with a fluorescent 25 marker, or with an enzymatic marker such as alkaline phosphatase or horseradish peroxidase to render them detectable. Conjugated avidin and antibodies are commercially available from companies such as Vector Laboratories (Burlingame, California) and Boehringer 30 Mannheim (Indianapolis, Indiana).

The enzyme can be detected through a colorimetric reaction by providing a substrate and/or a catalyst for the enzyme. In the presence of various catalysts, different colors are produced by the reaction, and these colors can 35 be visualized to separately detect multiple probes. Any substrate and catalyst known in the art may be used.

Preferred catalysts for alkaline phosphatase include 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) and nitro blue tetrazolium (NBT). The preferred substrate for horseradish peroxidase is diaminobenzoate (DAB).

5 The following examples are presented in order to more fully illustrate the preferred embodiments of the invention. They should in no way be construed, however, as limiting the broad scope of the invention.

Abbreviations:

10	ATP	-	adenosine triphosphate
	dATP	-	deoxyadenosine triphosphate
	CTP	-	cytidine triphosphate
	dCTP	-	deoxycytidine triphosphate
	GTP	-	guanosine triphosphate
15	dGTP	-	deoxyguanosine triphosphate
	dTTP	-	thymidine triphosphate
	UTP	-	uridine triphosphate
	NTP	-	nucleoside triphosphate
	nmole (nM)	-	nanomole
20	pmole (pM)	-	picomole
	mmole (mM)	-	millimole
	(μM)	-	micromole
	ng	-	nanogram
	μg	-	microgram
25	bis	-	bisacrylamide (N, N'-methylenebis-acrylamide)
	5'	-	the 5' position in the pentose
	3'	-	the 3' position in the pentose

30 EXAMPLE 1

Preparation of Target DNA

A 889 basepair region of the multidrug resistance gene (MDR-1) (Figure 4, SEQ ID NO:1) was selected as a target DNA for the system. The MDR-1 gene is available from the American Type Culture Collection, ATCC No. 65704. The target DNA was prepared by the standard polymerase chain

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reaction with

Primer A (SEQ ID. NO:2) 5'-AGGTTAGTACCAAGAGGGCTCTGG-3' and Primer B (SEQ ID NO:3) 5'-ACTAACAGAACATCCTCAAAGCTC-3' based on the known sequence of the gene. The PCR reaction 5 mixture comprised 1mM Tris HCl (pH 8.4), 5 mM KCl, 1.2 mM MgCl<sub>2</sub>, 0.8 mM of each dNTP, 1  $\mu$ M of Primer A, 1  $\mu$ M of Primer B, 1 ng of template DNA, 2.5 units of AmpliTaq<sup>TM</sup> DNA polymerase (Perkin Elmer Cetus Corporation, Norwalk, Connecticut). The reaction mixture was heated at 94°C for 10 6 min., and then put through the following cycle 30 times: 94°C for 1 min, 65°C for 45 sec., and 72°C for 3 min. The final polymerization was done at 72°C for 10 min.

20  $\mu$ g of DNA was digested with 40 units of RsaI restriction endonuclease at 37°C for 2 hours under the 15 conditions recommended for the enzyme. An aliquot was run on an agarose gel to confirm that the DNA was completely digested. DNA was then extracted sequentially with equal volumes of phenol, phenol-chloroform (1:1) and chloroform, and then precipitated with two volumes of ethanol. The DNA 20 pellet was suspended in deionized water and the concentration determined by measuring the optical density at 260 nm.

EXAMPLE 2

25 Preparation of Oligonucleotides

Deoxynucleotide oligomers were synthesized on Milligen/Bioscience Cyclone Plus DNA Synthesizers [Millipore Corporation, Bedford, Massachusetts] using beta-cyanoethyl phosphoramidite chemistry. All reagents for 30 oligonucleotide synthesis were purchased from Millipore Corporation [Bedford, Massachusetts].

Oligonucleotides having the following sequences were synthesized:

Oligo 1 (SEQ ID NO. 4):  
35 5' CAACATTTTC ATTTCAACAA CTCC 3'  
Oligo 2 (SEQ ID NO. 5):

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5' TTCTTTCTTA TCTTTCAGTG CTTGTCCAGA 3'

Oligo 3 (SEQ ID NO. 6):

5' GGAGTTGTTG AAATGAAAAT GTTGTC 3'

After the specified sequence had been assembled, a 60  
5 minute room temperature treatment with ammonium hydroxide  
was used to cleave the oligonucleotide from the support.  
The oligonucleotide was incubated with ammonium hydroxide  
at 55°C overnight treatment to remove the protecting  
groups. Ammonium hydroxide was evaporated to dryness in a  
10 Speedvac Concentrator (Savant Instruments, Inc.,  
Farmingdale, New York). The oligonucleotide was suspended  
in deionized water and extracted three times with an equal  
volume of water-saturated N-butanol. Any traces of  
N-butanol left were removed by evaporation in a Speedvac  
15 Concentrator. The concentration of oligonucleotide was  
determined by measuring optical density at 260 nm in a  
spectrophotometer.

EXAMPLE 3

20 Phosphorylation of oligonucleotides

Each oligonucleotide was phosphorylated at the 5' end  
with ATP and T4 polynucleotide kinase. The reaction  
mixture (100  $\mu$ l) contained 2 nmoles of each oligonucleotide  
50 mM Tris HCl pH 7.6, 10 mM MgCl<sub>2</sub>, 5 mM DTT, 0.1 mM  
25 spermidine hydrochloride, 0.1 mM EDTA, 1 mM ATP and 50  
units of T4 polynucleotide kinase (GIBCO BRL, Gaithersburg,  
Maryland). After 1 hour at 37°C, the enzyme was  
inactivated by heating at 65°C for 10 minutes.

30

EXAMPLE 4

<sup>32</sup>P Labeling of oligonucleotides

Oligonucleotides (20 pmoles) were labeled with <sup>32</sup>P at  
their 5' end in 60  $\mu$ l of 50 mM Tris HCl pH 7.6, 10 mM  
MgCl<sub>2</sub>, 5 mM DTT, 0.1 mM spermidine hydrochloride, 0.1 mM  
35 EDTA and 200  $\mu$ Ci of [ $\gamma$ -<sup>32</sup>P] ATP (3000 Ci/mmol = 67 pmoles

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of ATP; NEN Research Products Div. of Dupont, Boston, Massachusetts). The reaction was started by adding 20 units of T4 polynucleotide kinase (GIBCO BRL, Gaithersburg, Maryland) and incubated at 37°C for 1 hour. T4 5 polynucleotide kinase was heat inactivated at 65°C for 10 minutes.

EXAMPLE 5

Amplification of DNA

10 Oligonucleotides 1, 2 and 3 at a final concentration of 0.2  $\mu$ M were incubated in the presence or absence of target DNA (0.5 fmole =  $3 \times 10^8$  molecules) in 20  $\mu$ l of 25 mM Tris HCl pH 8.0, 10 mM KCl, 2 mM MgCl<sub>2</sub>, 10 mM DTT, 2 mM NAD<sup>+</sup> and 50  $\mu$ M of dATP, dGTP, dCTP and dTTP. The stock solution of 15 dNTP's was maintained at -20°C.

Three different experiments were performed. In each case, only one oligonucleotide was labeled. 15 units of Taq ligase (New England Biolabs, Beverly, Massachusetts) 20 and 1 unit of AmpliTaq<sup>TM</sup> DNA Polymerase (Perkin-Elmer Corporation, Norwalk, Connecticut) were added and the mixture was overlaid with a drop of mineral oil. Reactions were incubated in a single reaction medium in an Ericomp<sup>TM</sup> Thermal Cycler (Ericomp Incorporation, San Diego, 25 California) at 94°C for 6 minutes. The reaction mixture was incubated for 1 minute at 94°C, and 4 minutes at 65°C, with this cycle being repeated 30 times.

The product formation was followed independently using each <sup>32</sup>P-labeled oligonucleotide. The reaction was stopped 30 by adding 13  $\mu$ l of stop solution (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol FF). Samples were stored at -20°C until analyzed by electrophoresis.

35

EXAMPLE 6

Separation of the amplification products

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The products of the amplification reaction were separated on an 8% polyacrylamide gel (acrylamide:bis; 19:1) containing 8M urea in 100 mM Tris Borate pH 8.3, 2 mM EDTA. A BRL sequencing gel apparatus model S2 (BRL, 5 Gaithersburg, Maryland) was used to run the gel.

Samples (4  $\mu$ l) were denatured by boiling before loading on the gel. Electrophoresis was performed at a constant 60 watts for 2 hours. The gel was dried and exposed to a Phospho Screen<sup>TM</sup> (Molecular Dynamics, Sunnyvale, 10 California) and analyzed by a Phosphor Imager<sup>TM</sup> (Molecular Dynamics, Sunnyvale, California).

Figure 2 is a printout from a Phosphor Imager scan of the samples amplified by the method described in Example 5. In Lane 1 the reaction mixture contained labeled Oligo 1 15 and unlabeled Oligo 2 and 3. In Lane 2 the reaction mixture was the same as in Lane 1 with the addition of target DNA. The amplified DNA band is indicated with an arrow. In Lane 3 the reaction contained labeled Oligo 2 and unlabeled Oligo 1 and 3. In Lane 4 the reaction 20 mixture was the same as for Lane 3 with the addition of target DNA. In Lane 5 the reaction mixture contained unlabeled Oligo 1 and 2 and labeled Oligo 3. In Lane 6, the reaction mixture was the same as in Lane 5 with the addition of target DNA. It can be seen that amplification 25 does not occur in the absence of the target DNA and that amplification can be detected by labeling any of the oligonucleotides.

#### EXAMPLE 7

##### Embodiment 2

Deoxynucleotides are synthesized on Milligen/Bioscience Cyclone Plus<sup>TM</sup> DNA synthesizers (Millipore Corporation, Bedford Massachusetts) using beta-cyanoethyl phosphoramidite chemistry as described in Example 2. The 35 synthesis of oligonucleotides 1 and 3 was previously described in Example 2.

Oligonucleotides having the following sequences are synthesized:

Oligo 4 (SEQ ID NO:7)

5' GTTCGGAAAGT TTTCTATTGC TTCAGTAGCG 3'

5 Oligo 5 (SEQ ID NO:8)

5' CTACTGAAGC AATAGAAAAC TTCCGAAC 3'

The oligonucleotides are either phosphorylated at the 5' end with ATP and T4 polynucleotide kinase as described in Example 3 or labeled with  $^{32}\text{P}$  at their 5' end as described  
10 in Example 4.

The target DNA is prepared as described in Example 1.

Phosphorylated oligonucleotides at a final concentration of 0.2  $\mu\text{M}$  are incubated in the presence of target DNA (0.5 fmole =  $3 \times 10^8$  molecules) in 20  $\mu\text{l}$  of 25 mM Tris HCl pH  
15 8.0, 10 mM KCl, 2 mM MgCl<sub>2</sub>, 10 mM DTT, 2 mM NAD<sup>+</sup> and 50  $\mu\text{M}$  of dATP, dCTP, dGTP and dTTP. The stock solution of dNTP's are maintained at -20°C.

15 units of Taq ligase (New England Biolabs, Beverly, Massachusetts) and 1 unit of AmpliTaq<sup>TM</sup> DNA polymerase  
20 (Perkin-Elmer Corporation, Norwalk, Connecticut) are added and the mixture is overlaid with a drop of mineral oil. Reactions are incubated in a single reaction medium in an Ericomp Thermal Cycler<sup>TM</sup> (Ericomp Incorporation, San Diego, California) at 94°C for 6 minutes. Then the reaction  
25 mixture is incubated for 1 minute at 94°C, and 4 minutes at 65°C, with this cycle being repeated 30 times.

The reaction is stopped by adding 13  $\mu\text{l}$  of stop solution (95% v/v formamide, 20 mM EDTA, 0.05% w/v bromophenol blue, 0.05% w/v xylene cyanol FF). Samples are stored at -20°C  
30 until analyzed by electrophoresis.

The products of the amplification reaction are separated as described in Example 6.

Although only preferred embodiments of the invention are specifically illustrated and described above, it will be  
35 appreciated that many modifications and variations of the present invention are possible in light of the above

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teachings and within the purview of the appended claims without departing from the spirit and intended scope of the invention.

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## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

(i) APPLICANT: BHATNAGAR, SATISH K.  
GEORGE JR., ALBERT L.

(ii) TITLE OF INVENTION: METHODS FOR AMPLIFYING NUCLEIC ACID  
SEQUENCES

(iii) NUMBER OF SEQUENCES: 8

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(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk  
(B) COMPUTER: IBM PC compatible  
(C) OPERATING SYSTEM: PC-DOS/MS-DOS  
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:

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(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:

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(C) REFERENCE/DOCKET NUMBER: 020160-103

(ix) TELECOMMUNICATION INFORMATION:

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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 2726 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: double  
(D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GATTGAGAAA GCTGTCAAGG AAGCCAATGC CTATGACTTT ATCATGAAAC TGCCTCATAA	60
ATTTGACACC CTGGTTGGAG AGAGAGGGGC CCAGTTGAGT GGTCGGCAGA ACCAGAGGAT	120
CGCCATTGCA CGTGCCCTGG TTGCAACCC CAAGATCCTC CTGCTGGATG AGGCCACGTC	180
AGCCTTGGAC ACAGAAAGCG AAGCAGTGGT TCAGGTGGCT CTGGATAAGG CCAGAAAAGG	240
TCGGACCACC ATTGTGATAG CTCATCGTTT GTCTACAGTT CGTAATGCTG ACGTCATCGC	300
TGGTTTCGAT GATGGAGTCA TTGTGGAGAA AGGAAATCAT GATGAACCTCA TGAAAGAGAA	360
AGGCATTCTAC TTCAAACATTG TCACAATGCA GACAGCAGGA AATGAAGTTG AATTAGAAAA	420
TGCAGCTGAT GAATCCAAAA GTGAAATTGA TGCCTTGAA ATGCTTCAA ATGATTCAAG	480
ATCCAGTCTA ATAAGAAAAA GATCAACTCG TAGGACTGTC CGTGGATCAC AAGCCCAAGA	540
CAGAAAGCTT AGTACCAAAG AGGCTCTGGA TGAAAGTATA CCTCCAGTTT CCTTTTGAG	600
GATTATGAAG CTAAATTAA CTGAATGGCC TTATTTGTT GTTGGTGTAT TTTGTGCCAT	660
TATAAATGGA GGCCTGCAAC CAGCATTGCA AATAATATTT TCAAAGATTA TAGGGGTTTT	720
TACAAGAATT GATGATCCTG AAACAAAACG ACAGAATAGT AACTTGTGTT CACTATTGTT	780
TCTAGCCCTT GGAATTATTT CTTTATTAC ATTTTCCTT CAGGGTTCA CATTGGCAA	840
AGCTGGAGAG ATCCTCACCA AGCGGCTCCG ATACATGGTT TTCCGATCCA TGCTCAGACA	900
GGATGTGAGT TGGTTTGATG ACCCTAAAAA CACCACTGGA GCATTGACTA CCAGGCTCGC	960
CAATGATGCT GCTCAAGTTA AAGGGGCTAT AGGTTCCAGG CTTGCTGTAA TTACCCAGAA	1020
TATAGCAAAT CTTGGGACAG GAATAATTAT ATCCTTCATC TATGGTTGGC AACTAACACT	1080
GTTACTCTTA GCAATTGTAC CCATCATTGCA AATAGCAGGA GTTGTGAAA TGAAAATGTT	1140
GTCTGGACAA GCACTGAAAG ATAAGAAAGA ACTAGAAGGT GCTGGGAAGA TCGCTACTGA	1200
AGCAATAGAA AACTTCCGAA CCGTTGTTTC TTTGACTCAG GAGCAGAAGT TTGAACATAT	1260
GTATGCTCAG AGTTGCAGG TACCATAACAG AAACCTTTG AGGAAAGCAC ACATCTTGG	1320
AATTACATTT TCCTTCACCC AGGCAATGAT GTATTTTCC TATGCTGGAT GTTCCGGTT	1380
TGGAGCCTAC TTGGTGGCAC ATAAACTCAT GAGCTTGAG GATGTTCTGT TAGTATTTTC	1440
AGCTGTTGTC TTTGGTGCCA TGGCCGTGGG GCAAGTCAGT TCATTTGCTC CTGACTATGC	1500

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CAAAGCCAAA ATATCAGCAG CCCACATCAT CATGATCATT GAAAAAACCC CTTTGATTGA	1560
CAGCTACAGC ACGGAAGGCC TAATGCCGAA CACATTGAA GGAAATGTCA CATTGGTGA	1620
AGTTGTATTC AACTATCCC CCCGACCGGA CATCCCAGTG CTTCAGGGAC TGAGCCTGGA	1680
GGTGAAGAAG GGCCAGACGC TGGCTCTGGT GGGCAGCAGT GGCTGTGGGA AGAGCACAGT	1740
GGTCCAGCTC CTGGAGCGGT TCTACGACCC CTTGGCAGGG AAAGTGTGTC TTGATGGCAA	1800
AGAAATAAAG CGACTGAATG TTCAGTGGCT CCGAGCACAC CTGGGCATCG TGTCCCAGGA	1860
CCCCATCCTG TTTGACTGCA GCATTGCTGA GAACATTGCC TATGGAGACA ACAGCCGGT	1920
GGTGTACAG GAAGAGATCG TGAGGGCAGC AAAGGAGGCC AACATACATG CCTTCATCGA	1980
GTCACTGCCT AATAAATATA GCACTAAAGT AGGAGACAAA GGAACATCAGC TCTCTGGTGG	2040
CCAGAAACAA CGCATTGCCA TAGCTCGTGC CCTTGTTAGA CAGCCTCATA TTTTGCTTTT	2100
GGATGAAGCC ACGTCAGCTC TGGATACAGA AAGTAAAAG GTTGTCCAAG AAGCCCTGGA	2160
CAAAGCCAGA GAAGGCCGCA CCTGCATTGT GATTGCTCAC CGCCTGTCCA CCATCCAGAA	2220
TGCAGACTTA ATAGTGGTGT TTCAGAATGG CAGAGTCAG GAGCATGGCA CGCATCAGCA	2280
GCTGCTGGCA CAGAAAGGCA TCTATTTTC AATGGTCAGT GTCCAGGCTG GAACAAAGCG	2340
CCAGTGAAC TCTGACTGTAT GAGATGTTAA ATACTTTTA ATATTTGTTT AGATATGACA	2400
TTTATTCAAA GTTAAAAGCA AACACTTACA GAATTATGAA GAGGTATCTG TTTAACATT	2460
CCTCAGTCAA GTTCAGAGTC TTCAGAGACT TCGTAATTAA AGGAACAGAG TGAGAGACAT	2520
CATCAAGTGG AGAGAAATCA TAGTTTAAAC TGCATTATAA ATTTTATAAC AGAATTAAAG	2580
TAGATTTAA AAGATAAAAT GTGTAATTT GTTTATATT TCCCATTGG ACTGTAAC	2640
ACTGCCTTGC TAAAAGATTA TAGAAGTAGC AAAAAGTATT GAAATGTTG CATAAAAGTGT	2700
CTATAATAAA ACTAAACTTT CATGTG	2726

## (2) INFORMATION FOR SEQ ID NO:2:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 24 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

AGGTTAGTAC CAAAGAGGCT CTGG

24

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 24 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

ACTAACAGAA CATCCTCAAA GCTC

24

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 24 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

CAACATTTTC ATTTCAACAA CTCC

24

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

TTCTTTCTTA TCTTTCAGTG CTTGTCCAGA

30

## (2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 26 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GGAGTTGTTG AAATGAAAAT GTTGTC

26

## (2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

GTTCCGGAAGT TTTCTATTGC TTCAGTAGCG

30

## (2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 28 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

CTACTGAAGC AATAGAAAAC TTCCGAAC

28

WHAT IS CLAIMED IS:

1. A process for amplifying enzymatically a target nucleic acid sequence contained in a nucleic acid or a mixture of nucleic acids, comprising the steps of:
  - a) selecting the target nucleic acid sequence;
  - b) providing primers, said primers comprising a first primer which is substantially complementary to a first segment at a first end of the target nucleic acid sequence and a second primer which is substantially complementary to a second segment at a second end of the target nucleic acid sequence and whose 3' end is adjacent to the 5' end of the first primer and a third primer which is similar to the first end of the target nucleic acid sequence and which is substantially complementary to at least a portion of said first primer;
  - c) providing at least four different nucleotide bases;
  - d) hybridizing said first and second primers to the target nucleic acid sequence in a target dependent manner to form a primer-target complex;
  - e) ligating under conditions such that the adjacent 5' end of the first primer and the 3' end of the second primer will ligate to form a fused amplification product substantially complementary to said target nucleic acid sequence;
  - f) dissociating said fused amplification product from said target nucleic acid sequence;
  - g) hybridizing said third primer to said fused amplification product;
  - h) extending said third primer in the presence of the nucleotide bases under conditions such that an extended amplification product is formed substantially complementary to said fused amplification product; and
  - i) dissociating the extended amplification product from the fused amplification product.

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2. The process of Claim 1, wherein the target nucleic acid is single stranded.

5 3. The process of Claim 1, wherein steps (d) through (i) are repeated at least once.

4. The process of Claim 1, wherein the target nucleic acid is DNA.

10 5. The process of Claim 1, wherein the target nucleic acid is RNA.

15 6. The process of Claim 1, wherein step (e) is conducted in the presence of a ligating enzyme.

7. The process of Claim 6, wherein the ligating enzyme is T4 DNA ligase.

20 8. The process of Claim 6, wherein the ligating enzyme is stable at 0-95°C.

9. The process of Claim 8, wherein the ligating enzyme is Taq ligase.

25 10. The process of Claim 1, wherein step (h) is conducted in the presence of a polymerase.

30 11. The process of Claim 10, wherein step (h) is conducted in the presence of a polymerase selected from the group consisting of E. coli DNA polymerase I, Klenow fragment of E. coli DNA polymerase I, and T4 DNA polymerase.

35 12. The process of Claim 10, wherein step (h) is conducted in the presence of a polymerase which is stable

at temperatures of 0-95°C.

13. The process of Claim 12, wherein the agent for polymerization is Taq DNA polymerase.

5

14. The process of Claim 1, wherein the target nucleic acid sequence contains at least one deletion or mutation that causes a genetic disease.

10

15. The process of Claim 1, wherein the target nucleic acid sequence is contained in a pathogenic organism, virus or oncogene.

16. The process of Claim 1, wherein one of said primers  
15 comprises two or more different oligonucleotides, one of said oligonucleotides having a sequence exactly complementary to said target nucleic acid sequence.

20

17. The process of Claim 1, wherein the target nucleic acid is double stranded nucleic acid comprising a first and second strand wherein said first and second primers are substantially complementary to said first strand and said third primer is substantially complementary to said second strand and said first and second strands are dissociated  
25 prior to step (d).

30

18. The process of Claim 1, wherein each of the steps is conducted sequentially without isolation or purification of the products.

19. The process of Claim 18 wherein each of the steps is conducted in a single reaction medium.

35

20. The process of Claim 1, wherein the 5' end of the first primer comprises a phosphorothioate group.

21. The process of Claim 1, wherein the 3' end of the second primer comprises an arabinosyl nucleotide.

22. A process for detecting enzymatically a mutation or 5 an allele in a target nucleic acid sequence contained in a nucleic acid or a mixture of nucleic acids, comprising the steps of:

- a) selecting the target nucleic acid sequence;
- b) providing primers, said primers comprising a 10 first primer which is substantially complementary to a first segment at a first end of the target nucleic acid sequence and a second primer which is substantially complementary to a second segment at a second end of the target nucleic acid sequence and whose 3' end is adjacent 15 to the 5' end of the first primer and a third primer which is similar to the first end of the target nucleic acid sequence and which is substantially complementary to at least a portion of said first primer, wherein one of said primers comprises two or more different oligonucleotides,
- 20 one of said oligonucleotides having a sequence exactly complementary to said target nucleic acid sequence wherein each oligonucleotide is labeled with a different label;
- c) providing at least four different nucleotide bases;
- 25 d) hybridizing said first and second primers to the target nucleic acid sequence in a target dependent manner to form a primer-target complex;
- e) ligating under conditions such that the adjacent 5' end of the first primer and the 3' end of the second 30 primer will ligate to form a fused amplification product substantially complementary to said target nucleic acid sequence;
- f) dissociating said fused amplification product from said target nucleic acid sequence;
- 35 g) hybridizing said third primer to said fused amplification product;

h) extending said third primer in the presence of the nucleotide bases under conditions such that an extended amplification product is formed substantially complementary to said fused amplification product;

5 i) dissociating the extended amplification product from the fused amplification product; and

j) determining which labeled primer is contained within the fused amplification product or the extended amplification product.

10

23. A process for amplifying enzymatically a target nucleic acid sequence contained in a nucleic acid or a mixture of nucleic acids comprising the steps of:

a) selecting the target nucleic acid sequence;

15 b) providing primers, said primers comprising a first primer which is substantially complementary to a first segment at a first end of the target nucleic acid sequence and a second primer which is substantially complementary to a second segment at a second end of the target nucleic acid sequence said second segment being spaced a number of nucleotides from said first segment and a third primer which is similar to the first end of the target nucleic acid sequence and which is substantially complementary to at least a portion of said first primer;

20 c) providing at least four nucleotide bases;

d) hybridizing said first and second primers to the target nucleic acid sequence in a target dependent manner to form a primer-target complex;

25 e) extending a 3' end of the second primer in the presence of the nucleotide bases under conditions such that an extended second primer is formed wherein the 3' end of the extended second primer terminates at a base adjacent to a 5' end of the first primer;

f) ligating the ends of the first and second extended primers under conditions such that said first and said extended second primers will form a fused

amplification product substantially complementary to said target nucleic acid sequence;

g) dissociating said fused amplification product from said target nucleic acid sequence;

5 h) hybridizing said third primer to said fused amplification product;

i) extending said third primer in the presence of the nucleotide bases under conditions such that an extended amplification product is formed substantially complementary 10 to said fused amplification product; and

j) dissociating the extended amplification product from the fused amplification product.

24. The process of Claim 23, wherein steps (d) and (j) 15 are repeated at least once.

25. The process of Claim 23, wherein the target nucleic acid is double stranded nucleic acid comprising a first and second strand wherein said first and second primers are 20 substantially complementary to said first strand and said third primer is substantially complementary to said second strand and said first and second strands are dissociated prior to step (d).

26. The process of Claim 25, further comprising a 25 fourth primer wherein the fourth primer is substantially complementary to said second target nucleic acid strand and said fourth primer is substantially complementary to said second primer.

30 27. The process of Claim 26, wherein the nucleic acid is denatured by heating.

28. The process of Claim 23, wherein the nucleic acid 35 is DNA.

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29. The process of Claim 23, wherein the nucleic acid is RNA.

30. The process of Claim 23, wherein step (f) is 5 conducted in the presence of a ligating enzyme.

31. The process of Claim 30, wherein the ligating enzyme is T4 DNA ligase.

10 32. The process of Claim 30, wherein the ligating enzyme is stable at 0-95°C.

33. The process of Claim 32, wherein the ligating enzyme is Ampligase.

15 34. The process of Claim 23 wherein steps (e) and (i) are conducted in the presence of polymerase.

20 35. The process of Claim 34, wherein steps (e) and (i) are conducted in the presence of a polymerase selected from the group consisting of E. coli DNA polymerase I, Klenow fragment of E. coli DNA polymerase I and T4 DNA polymerase.

25 36. The process of Claim 34, wherein steps (e) and (i) are conducted in the presence of a polymerase which is stable at 0-95°C.

30 37. The process of Claim 36, wherein the polymerase is Tag DNA polymerase.

38. The process of Claim 23, wherein the target nucleic acid sequence contains at least one deletion or mutation that causes a genetic disease.

35 39. The process of Claim 23, wherein the target nucleic acid sequence is contained in a pathogenic organism, virus

or oncogene.

40. The process of Claim 23, wherein one of said primers comprises two or more oligonucleotides, one of said 5 oligonucleotides having a sequence exactly complementary to said target nucleic acid.

41. The process of Claim 23, wherein each of the steps is conducted sequentially without isolation or purification 10 of the products.

42. The process of Claim 41, wherein each of the steps is conducted in a single reaction medium.

15 43. The process of Claim 23, wherein the 5' end of the first primer comprises an phosphorothioate group.

44. The process of Claim 26, wherein the 5' end of the fourth primer comprises an phosphorothioate group.

20 45. A process for detecting enzymatically a mutation or an allele in a target nucleic acid sequence contained in a nucleic acid or a mixture of nucleic acids comprising the steps of

25 a. selecting the target nucleic acid sequence;  
b. providing primers, said primers comprising a first primer which is substantially complementary to a first segment at a first end of the target nucleic acid sequence and a second primer which is substantially complementary to a second segment at a second end of the target nucleic acid sequence said second segment being spaced from said first segment and a third primer which is similar to the first end of the target nucleic acid sequence and which is substantially complementary to at 30 least a portion of said first primer wherein one of said primers comprises two or more different oligonucleotides,  
35

one of said oligonucleotides having a sequence exactly complementary to said target nucleic acid sequence wherein each oligonucleotide is labeled with a different label;

5           c. providing at least four nucleotide bases;

5           d. hybridizing said first and second primers to the target nucleic acid sequence in a target dependent manner to form a primer-target complex;

10           e. extending a 3' end of the second primer in the presence of the nucleotide bases under conditions such that an extended second primer is formed wherein the 3' end of the extended second primer is adjacent to a 5' end of the first primer;

15           f. ligating the ends of the first and second primers under conditions such that said first and said extended second primers will form a fused amplification product complementary to said target nucleic acid sequence;

15           g. dissociating said fused amplification product from said target nucleic acid sequence;

20           h. hybridizing said third primer to said fused amplification product;

20           i. extending said third primer in the presence of the nucleotide bases under conditions such that an extended amplification product is formed complementary to said fused amplification product; and

25           j. dissociating the extended amplification product from the fused amplification product; and

25           k. determining which labeled primer is contained within the fused amplification product or the extended amplification product.

30

46. A kit for amplifying at least one target nucleic acid sequence contained in a nucleic acid or a mixture of nucleic acids, in accordance with Claim 1, comprising:

35           a. first, second and third primers;

35           b. a ligating enzyme;

35           c. a polymerizing enzyme; and

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d. at least four nucleotides.

47. The kit of Claim 46 further comprising:

5 e. a detectable marker attached to one of said  
primers.

48. The kit of Claim 46, further comprising:

e. a buffer suitable for the ligation and  
polymerization reactions.

10

49. A kit for amplifying at least one target nucleic  
acid sequence contained in a nucleic acid or a mixture of  
nucleic acids, in accordance with Claim 23, comprising:

15 a. first, second and third primers;  
b. a ligating enzyme;  
c. a polymerizing enzyme; and  
d. at least four nucleotides.

50. The kit of Claim 49 further comprising:

20 e. a detectable marker attached to at least one of  
said primers.

51. The kit of Claim 49, further comprising:

25 e. a buffer suitable for the ligation and  
polymerization reactions.

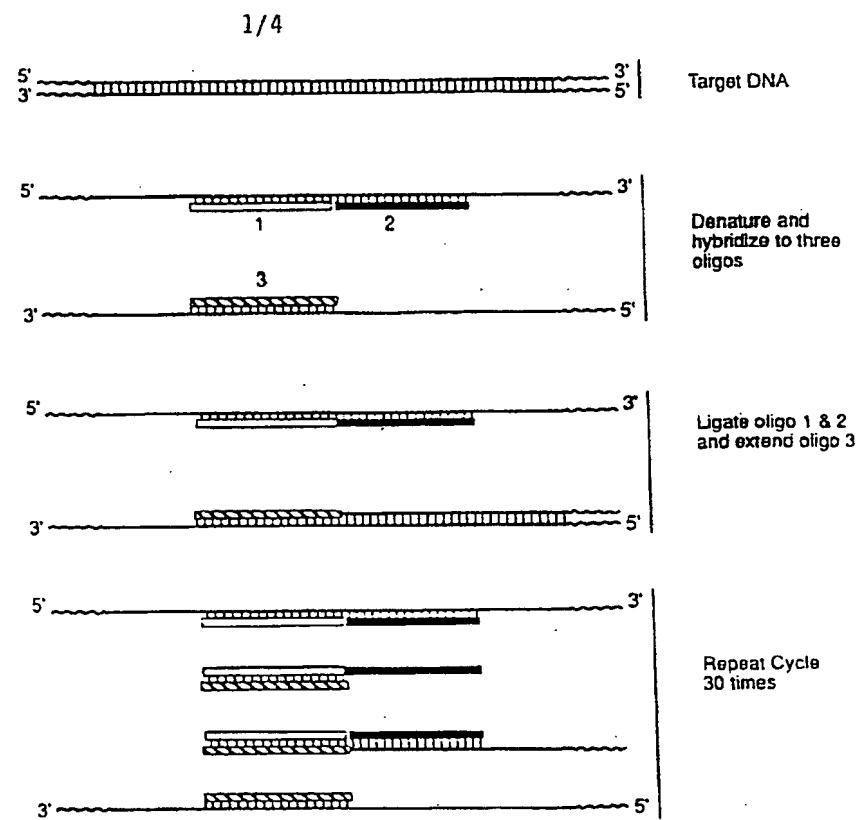
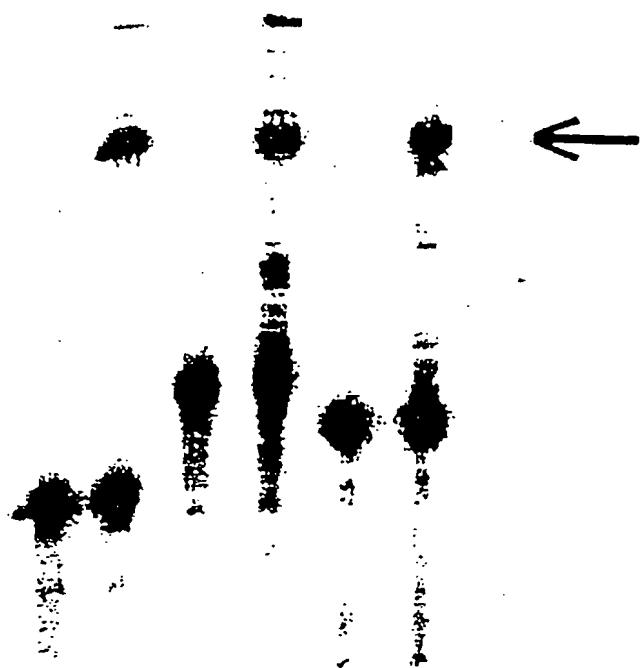


FIGURE 1

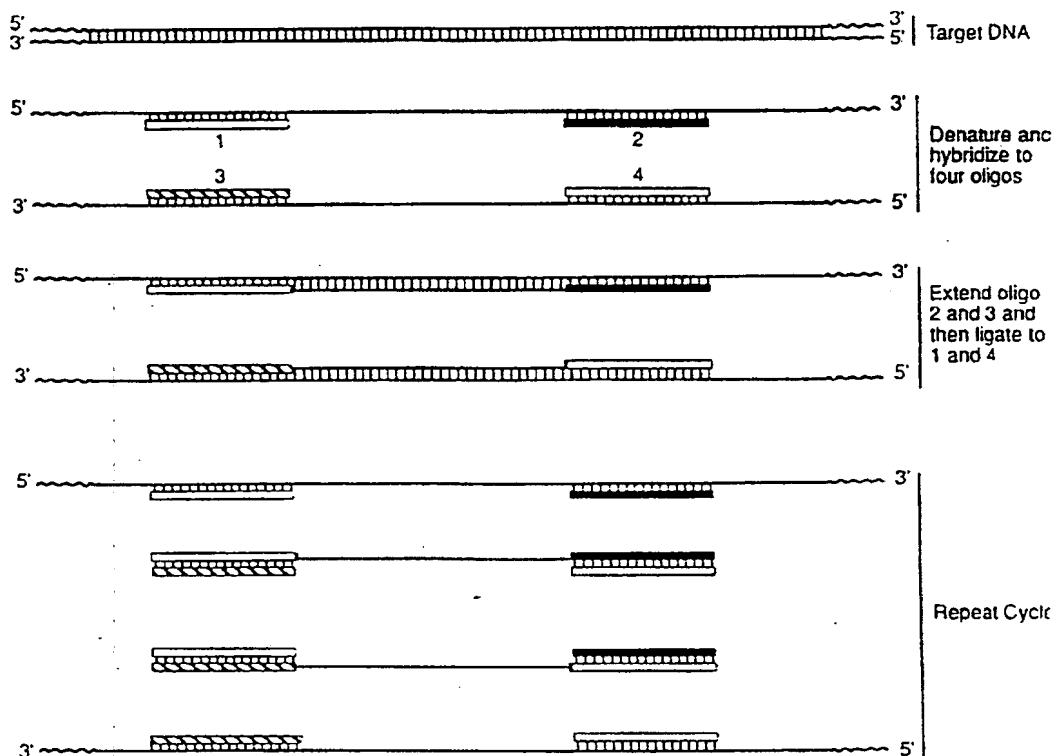
2/4

1 2 3 4 5 6

FIGURE 2



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## Modified OCR

FIGURE 3

1921 GATTGAGAAA GCTGTCAAGG AAGCCAATGC CTATGACTTT ATCATGAAAC TGCCTCATAA  
 1981 ATTTGACACC CTGGTTGGAG AGAGAGGGC CCAGTTGAGT GGTGGGCAGA AGCAGAGGAT  
 2041 CGCCATTGCA CGTGCCTCTGG TTGCGAACCC CAAGATCCTC CTGCTGGATG AGGCCACGTC  
 2101 AGCCTTGGAC ACAGAAAGCG AAGCAGTGGT TCAGGTGGCT CTGGATAAGG CCAGAAAAGG  
 2161 TCGGACCC ATTGTGATAG CTGATCGTT CTCTACAGTT CGTAACTCTG ACCTCATCGC  
 2221 TGGTTTCGAT GATGGAGTCA TTGTTGGAGA AGGAATCAT GATGAACTCAGA TGAAAGAGAA  
 2281 AGGCATTTAC TTCAAACCTG TCACAAATGCA GACAGCAGGA AATGAAGTTG AATTAGAAAA  
 2341 TGCAGCTGAT GAAATCCAAA GTGAAATTGA TGCTTGGAA ATGCTCTCAA ATGATTCAAG  
 2401 ATCCAGTCAT ATAAGAAAAA GATCAACTCG TAGGAGTGTG CGTGGATCAC AAGCCCAAGA  
 2461 CAGAAAGCTT AGTACCAAAAG AGGCTCTGGA TGAAAGTATA CCTCCAGTT CCTTTTGGAG  
 2521 GATTATGAAG CTAAATTTAA CTGAATGCCCT TTATTTTGTG TTGGGTGTAT TTGTTGCCAT  
 2581 TATAAAATGGA GGCCTGCAAC CAGCATTGCA AATAATATTT TCAAAGATTA TAGGGGTTTT  
 2641 TACAAGAATT GATGATCCTG AAACAAAACG ACAGAATAGT AACTTGTTTT CACTATTGTT  
 2701 TCTAGCCCTT GGAATTATTT CTTTTATTAC ATTTTCCCTT CAGGGTTCA CATTGGCAA  
 2761 AGCTGGAGAG ATCTTCACCA AGCGGCTCCG ATACATGGTT TTCCGATCCA TGCTCAGACA  
 2821 GGATGTCAGT TGGTTTGATG ACCCTAAAAA CACCACTGGA GCATTGACTA CCAGGCTCGC  
 2881 CAATGATGCT CCTCAAGTAA AAGGGGCTAT AGGTTCCAGG CTGCTGTAA TTACCCAGAA  
 2941 TATAGCAAT CTGGGACAG GAATAATTAT ATCTTCATC TATGGTTGGC AACTAACACT  
 3001 GTTACTCTTA GCAATTGATC CCATCATTGC AATAGCAGGA GTTGTGAAA TGAAATGTT  
 3061 GTCTGGACAA GCACTGAAAG ATAAGAAAGA ACTAGAAGGT GCTGGGAAGA TCGCTACTGA  
 3121 AGCAATAGAA AACTTCCGAA CGCTTGTTC TTGACTCAG GAGCAGAAGT TTGAACATAT  
 3181 GTATGCTCAG AGTTTGCAAG TACCATACAG AAACCTTTG AGGAAGGCA ACATCTTTG  
 3241 AATTACATTG TTCTTCACCC AGGCAATGAT GTATTTTCC TATGCTGGAT GTTTCCGGTT  
 3301 TGGAGCCTAC TTGGTGGCAC ATAAACTCAT GAGCTTGTGAG GATGTTCTGT TAGTATTTT  
 3361 AGCTGTTGTC TTGGTGTCCA TGGCCGTGGG GCAAGTCAGT TCATTTGCTC CTGACTATGCC  
 3421 CAAAGCCAAA ATATCAGCAG CCCACATCAT CTCATGTCATT GAAAAAACCC CTTTGATTGA  
 3481 CAGCTACAGC ACGGAAGGCC TAATGCCGAA CACATTGGAA GGAAATGTCATC CATTGGTGA  
 3541 AGTTGTATTTC AACTATCCCA CCCGACCGGA CATCCCACTG CTTCAAGGGAC TGAGGCTGGG  
 3601 GGTGAAGAAG GGCCAGACGC TGGCTCTGGT GGGCAGGAGT GGCTGTGGGA AGAGCACAGT  
 3661 GGTCCAGCTC CTGGAGCGGT TCTACGACCC CTGGCAGGG AAAGTGTGCA TTGATGGCAA  
 3721 AGAAATAAAAG CGACTGAATG TTCACTGGCT CGGAGCACAC CTGGGCACTCG TGTCCCAGGA  
 3781 GCCCATCTTG TTGACTGCA GCATTGCTGA GAACATTGCC TATGGAGACA ACACCCCCGGT  
 3841 GGTGTACAG GAAGAGATCG TGAGGGCAGC AAAGGAGGCC AACATACATG CTTTCATCGA  
 3901 GTCACTGCCT ATAAATATA GCACTAAAGT AGGAGACAAA GGAACCTCAGC TCTCTGGTGG  
 3961 CCAGAAACAA CGCATTGCCA TAGCTCGTC CCTTGTTAGA CAGCCTCATA TTTTGTTTT  
 4021 GGATGAAGCC ACGTCAGCTC TGGATACAGA AAGTAAAAG GTTGTCCAAG AAGCCCTGGG  
 4081 CAAAGCCAGA GAAGGGCCGA CCTGCATTGT GATGCTCAC CGCCCTGTCATC CCATCCAGAA  
 4141 TGCAGACTTA ATAGTGGTGT TTCAAGATGG CAGAGTCAG GAGCATGGCA CGCATCAGCA  
 4201 GCTGCTGGCA CAGAAAGGCC TCTATTTTC AATGGTCAGT GTCCAGGCTG GAACAAAGCG  
 4261 CCAGTGAACCTGACTGAT GAGATGTTAA ATACTTTTTA ATATTTGTTT AGATATGACA  
 4321 TTATTCAAA GTTAAAGCCA AACACTTACA GAATTATGAA GAGGTATCTG TTTAACATT  
 4381 CCTCACTGCA GTTCAGACTC TTCAAGACTC TCGTAATTAA AGGAACAGAG TGAGAGACAT  
 4441 CATCAAGTGG AGAGAAATCA TAGTTAAAC TGCAATTATAA ATTTTATAAC AGAATTAAAG  
 4501 TAGATTTAA AAGATAAAAT GTGTAATTTT GTTATATT TCCCATTTGG ACTGTAACIG  
 4561 ACTGCTTGC TAAAAGATTA TAGAAGTAGC AAAAGTATT GAAATGTTG CATAAAAGTGT  
 4621 CTATAATAAA ACTAAACTTT CATGTC

FIGURE 4

(SEQ. ID NO. 1)

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US93/10883

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :C12Q 1/68; C12P 19/34  
US CL :435/6; 435/91.2

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols).

U.S. : 435/6; 435/91.2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

MEDLINE, APS

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO, A, 90/01069 (SEGEV) 08 FEBRUARY 1990, see pages 5, 10-12.	1-51
Y	US, A, 4,683,195 (MULLIS ET AL.) 28 JULY 1987, see entire document.	1-51
Y	ERLICH ET AL., "POLYMERASE CHAIN REACTION" PUBLISHED 1990 by COLD SPRING HARBOR LABORATORY PRESS (COLD SPRING HARBOR, NY), pages 75-81, see entire document.	45

 Further documents are listed in the continuation of Box C.  See patent family annex.

Special categories of cited documents:		
"A"	document defining the general state of the art which is not considered to be part of particular relevance	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"E"	earlier document published on or after the international filing date	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubt on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means	"&" document member of the same patent family
"P"	document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search 22 February 1994	Date of mailing of the international search report MAR 17 1994
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer SCOTT HOUTTEMAN <i>Jill Warden for</i>
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